ZOOLOGICAL SCIENCE 11: 167–174 (1994)

REVIEW

Insulin-like Growth Factors: Growth, Transgenes and Imprinting

ANDREW WARD[1], PÄR BIERKE[2,3], EVA PETTERSSON[2], WILHELM ENGSTRÖM[2]*

[1]*Cancer Research Campaign Growth Factors, Department of Zoology, University of Oxford, South Parks Road, Oxford OX13PS, UK and* [2]*Department of Pathology Faculty of Veterinary Medicine Swedish University of Agricultural Sciences S-750 07 Uppsala, Sweden* [3]*National Defenee Research Institute, S-90182 Umeå Sweden*

I BACKGROUND

The idea of integrating stable gene mutations into the germ line has long fascinated developmental biologists. Experimental gene modifications that influence subsequent developmental patterns have therefore been at the top of the list of priorities. The field of experimental embryology took off in the early 1960s when Krystof Tarkowski [86] and Beatrice Mintz [59] succeeded in fusing genetically distinct 8-cell embryos into stable chimaeras. In 1968 Richard Gardner [28] was able to show that by moving the inner cell mass from one embryo to another a chimaera could be formed. But it was not until 1974 when Jaenisch and Mintz [41] could report the first deliberate genetic modification of the mouse embryo. By infecting embryos at the blastocyst stage with SV40-virus they were able to demonstrate a stable integration of viral DNA in the live born progeny. This breakthrough was rapidly followed by Moloney murine leukemia virus mediated germ line transfer of foreign nucleic acid sequences [42]. Since that time the field has exploded, it appears pertinent to briefly discuss some key experimental landmarks during the last 20-year period.

It was in 1980 [30] that the pronuclear microinjection technique was devised and used to demonstrate the possibility of inserting foreign DNA from almost any source into the murine embryo. This technique can be applied to almost any animal species. The next important step forward was the demonstration that integrated foreign DNA can also be expressed. In 1981 Palmiter *et al.* [64] were able to ligate the 5′ regulatory sequence of the murine metallothionein (MT1) gene with the core sequence of the herpes virus thymidine kinase gene. This transgene was injected into pronuclei and properly integrated. In the adult progeny, the herpes thymidine kinase gene was expressed in a pattern as expected from the MT1 gene with abundant expression in kidney and liver. Furthermore, it was concluded from this and subsequent studies that the actual integration site plays an inferior role in determining the level of expression. It is rather the cis-acting regulatory sequences in and adjacent to genes that determine spatial and temporal transcription patterns [32, 63]. There are however important exceptions to this rule. In the case of the beta globin gene the absence of key enhancer elements makes it relatively more dependent on the integration site for adequate expression [13].

This implies that the interplay between structural regulatory elements is imperative in determining stage- and tissue-specific expression. If an isolated promoter sequence is fused with a heterologous coding region and integrated in the germ line, the expression pattern will in many cases differ from that of an integrated genomic clone containing the regulatory and structural elements in intact order. There are also transacting factors that affect expression of integrated foreign genes. The presence or absence of such transactivators indeed differ between species. When a human gene is integrated into transgenic mice, the transgene may therefore be expressed in organs that do normally not express the murine counterpart, but which are sites of expression in man.

Foreign genes normally integrate as head to tail concatamers into one unique site in the mouse genome. However at this single site a considerable number (from one to 50 copies) of transgenes can be integrated. There is ample evidence that this large scale integration is not always a straight forward process. It predisposes delays and recombinations leading to mosaic patterns. It can also lead to strange structural alterations of the mouse host genome including deletions, duplications, translocations and interspersion of islands of genomic DNA within the transgene sequences. The disruption of endogenous gene function upon transgene integration is also an alarmingly frequent event [58]

II METHODOLOGY

1. Pronuclear injection

Broadly, two approaches are currently employed in generating transgenic mice, leading to either over-expression or targeted alteration of the gene of interest. In the first approach, exogenous copies of the gene are incorporated into

Received February 10, 1994
* Reprint requests should be addressed to Dr. W. Engström.

the genome with the aim of elevating its level of expression *in vivo*. This is usually achieved by injecting fertilised eggs, collected approximately 12 hr after mating has taken place, with linear cloned DNA fragments [39]. The injected embryos are then introduced into the uterus of receptive, pseudopregnant females, so that normal development can proceed. Liveborn mice are tested for the presence of the transgene by assaying biopsy material using DNA (Southern) blotting or polymerase chain reaction (PCR) techniques. Although the proportion of transgenic offspring which result is very much dependant on the nature of the injected DNA construct, frequencies of transgenesis over 20% can be achieved, representing one major advantage of this approach. This is offset against the principle disadvantage of this scheme, in that incorporation of the transgene into the genome occurs at essentially randomly determined positions. Since expression of the transgene can be influenced by sequences surrouding the integration site (so called position effects) and since transgene insertion can disrupt the function of a resident gene, the phenotype associated with any given DNA construct must be qualified by studying lines of transgenic mice representing at least two independent integration events.

2. Embryonic stem cells

In the second approach to producing transgenic mice, more recently developed technology allows the targeted alteration of specific endogenous genes by homologous recombination in embryonic stem (ES) cells. ES cells are derived from blastocyst embryos and can be maintained in culture for many generations before being microinjected into host blastocysts and returned to pseudopregnant females. Subsequently, they can colonise all tissues of the resulting animals, which develop to form chimeric mice. Chimeric animals in which the ES cells have given rise to germ cells can be bred to produce transgenic offspring which are entirely ES cell derived [40, 43]. Compared with the one cell embryos used for pronuclear microinjection, ES cells represent a more abundant source of starting material which can be manipulated *in vitro* over a much longer time period, greatly increasing the scope for the manipulation of the genome. This is essential to the success of the gene targeting scheme. Gene targeting requires a construct comprising sequences of the target gene, bearing a mutation which will alter or ablate its function, together with at least one selectable marker gene (such as neo^r, which confers resistance to the drug G418). Often the neo^r gene is positioned within the construct such that it disrupts or replaces part of the coding region of the target gene. This construct can be introduced rapidly into many thousands of ES cells by electroporation. These cells are subsequently grown in the presence of G418 to identify resistant colonies which represent those cells in which the targeting construct was stably incorporated into the genome. The sequences of the target gene which flank the neo^r gene provide regions of homology which allows recombination to take place such that the cloned DNA can replace the target gene following its introduction into embryonic stem cells. However, since homologous recombination events are usually much less frequent than random integration events, the G418 resistant ES cell colonies must be screened to establish which should be used to generate chimeras. This screening process is one of the rate limiting steps of this transgenic route since the homologous recombination events typically represent less than 0.1% of all stable transformants. However, this figure is constantly being improved with the development of methods to enrich for homologous recombination over random integration. These improvements include the use of a second selectable marker which must be lost from the targeting vector during homologous recombination to allow cell survival [53] and the use of DNA within the targeting construct which derives from the same mouse strain as the host ES cells [87, 88].

3. Strategies for increased expression

Perhaps the crudest overexpression strategy relies upon the use of an entire genomic sequence which contains the full coding sequence with cognate upstream regulatory elements as the transgene. This approach has had a practical advantage in compensating for defects in mutant animals lacking a specific gene. A classical experiment succeeded in restoring fertility in hypogonadal mice by introducing the gonadotropin releasing factor gene [55]. In addition to its simplicity, this method can be advantageous where it is important to reproduce the expression patterns of the gene of interest. For instance following cloning of the sex-determining gene Sry, its own regulatory sequences were relied upon to create transgenic mice which were XX males [46]. In this case accumulated molecular and genetic evidence indicated that the testis determining gene was normally expressed transiently in specific cells in the developing gonad [47].

A more widely appreciated approach is based upon the fusion of a coding sequence with a well characterized promoter sequence that operates in a multitude of tissues. Commonly used promoters that induce widespread expression are the actin, H2 and SV40 promoters. Other promoter sequences induce expression in a more limited spectrum of cells and tissues. The mouse Mammary Tumour Virus Long Terminal Repeat (MMTV-LTR) operates mainly in secretory epithelial cells, and as a result transgenes containing this promoter sequence are expressed specifically in this cell type. When the MMTV-LTR was fused with the Granulocyte Macrophage Colony Stimulating Factor gene a strange subset of phenotypes were observed amongst the offspring [48]. Other promoters that allow cell specific expression include the Immunoglobulin promoter. When this sequence was fused with the interleukin-6 (IL-6) gene, mice harbouring this transgene developed plasmocytosis, with IL-6 expressing B-cells circulating throughout the body giving rise to high circulating levels of IL-6 protein [82].

The options can be further narrowed by using tissue specific promoters. The first of its kind to be utilized for this purpose was the rat insulin promoter (RIP) which is only

active in pancreatic beta cells. By fusing RIP with a nerve growth factor (NGF) coding sequence a unique pattern of hyperinnervation of the islets of Langerhans was achieved [20]. The great advantage of this approach is that the phenotypic effects on a single organ is enabled. Now a plethora of organ specific promoters are used for the purpose of site directed expression of transgenes. The bovine keratin 10 promoter directs the expression of transgenes to the supra basal layer of the skin and to the forestomach [2]. Gene expression can be directed to the mammary gland by using either the Whey Acidic Protein (WAP) promoter [35] or the beta lactoglobulin promoter [91]. The latter promoter limits expression to the secretory epithelium of the mammary gland. Moreover, induction of beta lactoglobulin expression coincides with that of beta casein in the tissue, indicating that the pattern of expression is determined by the differentiated state of the mammary cells [35, 91]. Using this promoter, protein products of the transgene can be secreted into milk and form up to 10% of the total milk protein or 30% of whey protein [1]. To achieve kidney specific expression, much attention has been devoted to the three murine renin genes Ren-IC, Ren-1d and Ren-2. However, the three renin genes exhibit distinct expression profiles at a number of extra renal sites [27, 75, 76].

III TRANGENIC STUDIES INVOLVING INSULIN AND THE INSULIN-LIKE GROWTH FACTORS

Insulin and the insulin-like growth factors (IGF-I and IGF-II) are structurally related and share affinities for the same set of trans-membrane receptors (reviewed in [72]). Each factor exhibits a preference for the appropriately named receptor (insulin, IGF type 1 and IGF type 2) but can interact with the others. This family of growth factors and receptors represent probably the most intensively studied using mouse transgenesis; insulin, IGF-I and IGF-II have all been analysed using overexpression strategies and IGF-I, IGF-II and the IGF type 1 receptor have each been the subject of gene targeting experiments to disrupt their function in mice. These experiments have revealed much about the *in vivo* functions of these factors and collectively they amply demonstrate the effectiveness of applying transgenic techniques to the analysis of mammalian growth factors.

1. Insulin

In several reports a common strategy has been adopted to elevate insulin expression at the natural sites of synthesis [11, 26, 54, 74]. By introducing copies of the human insulin gene into mice it was possible to distinguish mRNA and protein of transgene and endogenous origin while harnessing the cognate promoter to ensure expression was obtained in the pancreatic islets. The efficacy of this approach is clear since in all cases transgene expression was restricted to the pancreas and in one case, by comparing various lengths of promoter sequence, it was established that as little as 168 bp upstream of the transcript initiation site are sufficient to ensure correct tissue-specificity of expression from the insulin gene promoter [26]. In mice with detectable levels of human insulin, but no significant increase in total serum insulin levels, appropriate regulation of the human insulin transgene was established by challenging mice with glucose, amino acids or the hypoglycemic drug tolbutamide [11, 74]. In these cases normal pancreatic histology was reported and there were no obvious effects on growth. More recently it was shown that higher than normal serum insulin levels can result from similar transgenic experiments, consequently these mice are hyperglycemic in response to a glucose challenge and their normal fasting glucose levels suggest some insulin tolerance [54]. To date, however, transgenic experiments have not suggested any direct effects on growth *in vivo,* although insulin can promote growth *in vitro* [79] and is a routine addition in culture media. While it may be that insulin has no significant effect on growth *in vivo* further experiments, perhaps involving the targeted mutation of the insulin gene or its overexpression at ectopic sites and during earlier developmental stages, might be more revealing in this context.

2. IGF-I

A key role of IGF-I, in mediating the effects of growth hormone (GH) on postnatal growth, was proposed over ten years ago when adminstration of purified IGF-I was shown to stimulate growth in hypophysectomised rats [70]. This relationship was subsequently bome-out by experiments in which GH or IGF-I over-expression was achieved in transgenic mice. When rat or human GH genes were placed under the control of the mouse metallothionein I (mTM-I) gene promoter serum GH levels could be raised several hundred-fold, typically resulting in a 1.5-fold increase in adult body weight [64, 65]. Growth enhancement was detectable from about 3 weeks of age and was associated with elevated levels of circulating IGF-I. The rise in serum IGF-I was shown to preceed the acceleration in growth by about one week [56]. Although these data are consistent with the proposed role for IGF-I, the findings of Stewart *et al.* [80] suggest the increased circulating IGF-I might not be necessary to mediate GH action. These workers expressed human GH in mice using a mammary tumour virus LTR and these transgenic mice displayed supernormal growth kinetics similar to those described previously but did not always have high serum IGF-I levels. They suggest the extra growth results from direct GH action through the somatogenic and PRL receptors. Alternatively, stimulation of IGF-I production local to its sites of action might be the important factor as this could occur independently of changes in serum levels. In fact a combination fo direct and IGF-I mediated mechanisms of GH action remains likely and is consistent with the results of IGF-I over-expression in transgenic mice [55]. Although only one of two lines of mice established using an mMT-1/hIGF-I construct were found to express the introduced gene these mice were 1.3 fold larger than controls, which is within the range of the effect obtained with mMT-I/GH constructs.

However, the phenotype of the IGF-I transgenic mice differed from that of GH trangenics, notably in the range of organs which were enlarged or exhibited histopathological changes, and that the onset of the effect on live weights was delayed until at least six weeks after birth [7, 16, 57, 66].

Mice lacking a functional IGF-I gene have recently been created by gene targeting [3, 52]. The homozygous mutants were approximately 60% normal size at birth and their survival into adulthood varied with genetic background. Postnatally, the surviving animals continued to grow slowly, eventually attaning only about 30% the weight of normal littermates. These experiments confirm unequivocally that IGF-I has a significant effect on growth during embryogenesis, before any effects of GH are manifest.

3. IGF-II

In contrast with studies involving IGF-I, attempts to produce mice over-expressing IGF-II using promoters with relatively broad fields of activity have been largely unsuccessful. At least three groups of researchers found that, at best, only low levels of transgene expression were obtained when using promoters from the mMT-I (expression reported in 2/17 transgenic lines (63)), rat IGF-II (0/4; (49)) and human H-2k (0/8; (21)) genes. In all of these cases no significant effects on growth were reported. This can be attributed to an embryonic lethal effect which is thought likely to result from the presence of excess IGF-II, perhaps at critical sites and/or periods during development. Recent genetic evidence supports this theory as mice lacking the type 2 IGF receptor (T^{hp} mutant mice) usually die at mid-gestation, but this phenotype is rescued when they also lack a functional *Igf-2* gene [25]. The principle role for the type 2 receptor seems to be one of targeting its ligands (IGF-II and mannose-6-phosphate) for lysosomal degradation, and not mitogenic signalling. Thus, the lethality observed in T^{hp} mice is seen as an absence of this important regulator of IGF-II action.

Very recently the problems associated with producing IGF-II transgenic mice were circumvented by using a keratin gene promoter which had previously provided tissue restricted expression of a *h-ras* transgene [2]. IGF-II transgene expression was seen in all of the transgenic lines established using this promotor (Ward *et al.*, unpublished). These mice exhibited dramatic local effects on growth, consistent both with the paracrine/autocrine mode of action suggested for IGF-II by *in situ* expression studies (reviewed in [90]) and with proposed roles for IGF-II in growth related diseases, including Beckwith-Wiedemann syndrome and certain cancers [51, 73, 89]. These over-expression experiments complement studies of mice in which the IGF-II gene was disrupted by gene targeting.

Growth of IGF-II null mice was compromised during development, but not postnatally as recorded for mice without an intact IGF-I gene, and their size at birth was about 60% that of their normal littermates. Further differences between IGF-I and IGF-II null mice demonstrated that IGF-II acts at least two embryonic days earlier than IGF-I and that only IGF-II has an effect on placental growth [3, 73, 89]. The type 1 receptor was also mutated as part of this spectacular series of experiments and IGF-I-R null mice were more severely dwarfed at birth than either of those lacking individual functioning IGF genes. The availability of these three types of gene targeted mice, together with T^{hp} mutants, allowed the breeding of various double mutants [3, 25, 52]; some of their characteristics are summarized in Table 1. Comparison of all the resulting single and double mutants yeilded several important conclusions, including that IGF-I acts solely through the type 1 receptor (since the phenotypes of mice null for either *Igf-1r* or *Igf-1* and *Igf-1r* were indistinguishable) while IGF-II acts through both the type 1 receptor and at least one other (since similar phenotypes were

Table 1. Gross effects on growth of mice null for IGF and/or IGF receptor functions

Nullitype	Birth weight (% normal)	Placenta	Onset	Notes
Igf-1	60%	100%	E13.5	Survival to adulthood varies with genetic background. Some distortion in proportionality of features. Delays in ossification.
Igf-2	60%	75%	E11	Proportionally dwarfed. Delays in ossification.
Igf1r or Igf-1/Igf1r	45%	100%	E11	Neonatal death (respiratory failure). Marked hypoplasia in muscle and skin. Extended delays in ossification.
Igf1r/Igf-2 Igf-1/Igfr-2	30%	75%	E11	Neonatal death (respiratory failure). Marked hypoplasia in muscle and skin. Extended delays in ossification.
Igf2r	—	—	—	Mid-gestational lethality.
Igf-2/Igf2r	60%	75%	—	Neonatal lethality.

These results are implicit and all quantities are approximate. Data from (3, 14, 15, 25, 52). Note that Igf-2 results were obtained with heterozygous mutations, following paternal transmission of the null allele and the intact maternal allele is known to be expressed in some tissues.

displayed by mice null for either *Igf-1* and *Igf-2* or *Igf-1r* and *Igf-2* and in both cases these phenotypes were more severe than that of *Igf-1r* mutants). The identity of the second IGF-II receptor remains ambiguous, although it could be the insulin receptor, and this receptor was deemed the sole mediator of IGF-II action on placental growth.

IV TRANSCRIPTION FACTORS

One alternative approach to studying growth factor production *in vivo*, involves manipulation of relevant transcription factor genes and introducing them into transgenic animals. It has been suggested that many growth factor defects arise as a consequence of a defect in transcriptional regulation rather than mutations in the growth factor gene itself. This is particularly important in embryogenesis, and it was recently shown that deregulation of the pax-2 gene had a dramatic impact on kidney development [19]. This gene belongs to a family of transcription factors that display organ and stage specific activity. *pax-2* is expressed in the embryonic kidney after mesenchymal induction, in the ureter epithelium and early epithelial structures [17]. Furthermore, its expression is repressed upon terminal differentiation but persists at increased levels in Wilms tumours [18]. In this respect it mirrors the transcriptional activity of the IGF II gene which makes studies of persistent *pax-2* gene expression in transgenic animals particularly interesting. Deregulated *pax-2* expression in transgenic mice gave rise to histologically abnormal and dysfunctional renal epithelium reminiscent of the congenital nephrotic syndrome. The interrelationship between *pax-2* and IGF II and their combined detrimental impact on development however remains to be further clarified.

It has been shown that both the IGF-I and IGF-II genes contain AP-1 binding sites [12, 45]. Therefore studies which aim at altering the availability of Fos and Jun proteins ought to influence the expression of the IGF genes and would be of particular interest from a developmental point of view. When both copies of *c-jun* were inactivated by homologous recombination, perfectly viable ES-cells were obtained [37]. When these were integrated into the germ line it was found that heterozygous mutant mice were normal, but embryos lacking *c-jun*, died at mid or late gestation, and displayed impaired hepatogenesis, altered fetal liver erythropoesis and generalized oedema. Moreover, it was found that *c-jun* -/- ES cells were capable of participation in development of all somatic cells in chimaeras except liver cells [38]. An example of the opposite approach, namely an overexpression of the AP-1 component fos yielded an unexpected series of results. Expression of *c-fos* has for more than a decade been considered an early step in the chain of events between growth stimulation and onset of DNA-synthesis. However, embryonic development is a fine balance between proliferation, differentiation and programmed cell death. By studying *fos*-lacZ transgenic mice it was possible to demonstrate that continuous *fos* expression begins hours or days before the morphological demise of a condemned cell. Expression, therefore appears to be a hall mark of terminal differentiation and a harbinger of death [77]. These examples show that alteration of transcription factors expression lead to dramatic biological effects in transgenic animals. Therefore they should be taken into account when growth factors are to be considered as prime candidates for developmental processes in transgenic animals.

V GROWTH FACTORS, TRANSGENESIS AND GENOMIC IMPRINTING

One surprising outcome of the IGF-II gene knock-out experiments was the finding that the growth deficient phenotype occurred in heterozygous mutant mice when the mutation was inherited from a male but not from a female [15]. This led to the discovery that IGF-II is subject to genomic imprinting, with only the allele inherited from the male being expressed in most tissues [14]. The phenomenon of genomic imprinting was known before this, since nuclear transplantation experiments had demonstrated the nonequivalence of the male and female genetic contribution in mammalian development [84, 85], but *Igf-2* was the first gene known to be influenced by this peculiar form of regulation. Soon afterwards the *Igf-2r* gene was also shown to be imprinted after it was mapped within deletions of chromosome 17 harboured by the T^{hp} and T^{lub2} mutants [4]. In this case, in accord with the lethality associated with female but not male transmission of the T^{hp} and T^{lub2} mutations, the maternal allele was found to be active.

Opposite imprinting of the *Igf-2* and *Igf-2r* genes immediately brought to life an hypothesis of parent-offspring conflict predicted in plant and animal species in which the growth of progeny is dependent on a maternal nutient supply (e.g. the mammalian placenta) and in which multiple paternity is likely among offspring of individual females [45]. Under these circumstances, and given that larger offspring are the most well equipped to survive, the interest of the father is best served if each of his progeny are as large and strong as possible. As there is a chance that he may be usurped the father makes the most of each opportunity to reproduce although this might involve an increased burden on the mother. Obviously, she is guaranteed to be equally related to each of her offspring, irrespective of any changes in mate choice and it follows that it is in the mothers interest to distribute resources evenly among her unborn young whether within a litter or in conserving the capacity to produce later litters. Since this parental conflict of interest requires that a "choice" is made regarding growth during embryonic life, Haig and Westoby [34] suggested that this would be reflected at the molecular level. That *Igf-2* and the *Igf-2r* can be viewed as molecular weapons in this conflict is entirely in keeping with their antagonistic effects on growth and, of course, that it is respectively the paternally- and maternally-derived copies of each that are expressed [33].

Genomic imprinting of human IGF-II was recently con-

firmed [29] and is implicated in the ontogeny of the fetal overgrowth disease Beckwith Wiedemann syndrome [36]. Furthermore, it has been suggested that mis-regulation of imprinted genes might be important in a range of neoplastic diseases since, for instance, as only one allele is normally expressed then a single mutation would effectively result in a nullisomy. Conversely, activation of the normally silent allele would result in the gene product being overrepresented [23, 44]. Indeed relaxation of imprinting was recently demonstrated for IGF-II in a proportion of Wilms' tumours [60, 67].

There is one further intriguing link between IGF-II and imprinting in that the *H19* gene, located only about 80 kb from IGF-II [92], is also imprinted with the maternally derived copy being active in mouse and man [6, 93]. This gene has no ascribed function *in vivo* and might exert its effect as an RNA product since it does not encode an open reading frame [8]. However, the influence of *H19* on development is evidenced by transgenic experiments in which overexpression of intact H19 genes resulted in mid-gestational lethality [10]. It has been suggested that the regulation of IGF-II and H19 might be linked in some fashion since these two oppositely imprinted genes are such near neighbours [5, 83, 92]. The perceived importance of genomic imprinting in both normal development and growth related disease has fuelled an intense interest in achieving an understanding of the mechanism which underlies this phenomenon. An epigenetic signal which can be appropriately modified during passage of genes through either the male or female germline is required to establish and maintain allele specific expression of imprinted genes. Studies of the *Igf-2* [69], *Igf-2r* [81] and H19 [5, 24] genes indicate that DNA methylation might be involved in this process, and this was recently confirmed through the discovery that all three loci were lost in mice with a methyltransferase gene deficiency [50]. However, the race towards a full understanding is far from over. Early evidence suggests that not all of the signals required for properly imprinted expression have yet been discovered as only a minor proportion of *Igf-2* [49] and *H19* [5] transgenes behave in accord with their parent of origin, but it is without doubt that transgenesis will play a key role in unravelling this biological conundrum.

ACKNOWLEDGMENTS

The authors have been generously supported by the Cancer Research Campaign of Great Britain as well as by Cancerfonden and Barncancerfonden, Sweden.

REFERENCES

1 Archibald AL, McClenaghan M, Hornsey V, Simons JP, Clark AJ (1990) High levels of expression of biologically active human alpha1-antitrypsin in the milk of transgenic mice. Proc Natl Acad Sci USA 87: 5178–5182

2 Baillcul B, Surani MA, White S, Barton SC, Brown K, Blessing M, Jorcano J, Balmain A (1990) Skin hyperkeratosis and papilloma formationin transgenic mice expressing a ras oncogene from a suprabasal keratin promoter. Cell 62: 697–728

3 Baker J, Liu J, Robertson LJ, Efstriatiadis A (1993) Role of insulin-like growth factor type 2 receptor in embryonic and postnatal growth. Cell 75: 73–82

4 Barlow DP, Stoger R, Herrmann BG, Saito K, Schweifer N (1991) The mouse insulin-like growth factor type-2 receptor is imprinted and closely linked to the Tme locus. Nature 349: 84–87

5 Bartolomei MS, Webber AL, Brunkow ME, Tilghman SM (1993) Epigenetic mechanisms underlying the imprinting of the mouse H19 gene. Genes Dev 7: 166–167

6 Bartolomei MS, Zemel S, Tilghman SM (1991) Parental imprinting of the mouse H19 gene. Nature 351: 153–155

7 Behringer RR, Lewin TM, Quaife CJ, Palmiter RD, Brinster RL, d'ErcoLe AJ (1990) Expression of insulin-like growth factor I stimulates normal somatic growth in growth hormone-deficient transgenic mice. Endocrinology 127: 1033–1040

8 Brannan CI, Dees EC, Ingram RS, Tilghman SM (1990) The product of the H19 gene may function as an RNA. Mol Cell Biol 10: 28–36

9 Brinster RL, Chen HY, Trumbauer ME, Senear AW, Warren R, Palmiter RD (1981) Somatic expression of herpes thymidine kinase in mice following injection of a fusion gene into eggs. Cell 27: 223–231

10 Brunkow ME, Tilghman SM (1991) Ectopic expression of the H19 gene in mice causes prenatal lethality. Genes Dev 5: 1092–1101

11 Bucchini D, Ripoche M-A, Stinnakre M-G, Desbois P, Lores P, Monthioux E, Absil J, Lepesant J-A, Picter R, Jami J (1986) Pancreatic expression of human insulin gene in transgenic mice. Proc Natl Acad Sci USA 83: 2511–2515

12 Caricasole A, Ward A (1993) Transactivation of mouse IGF II gene promoters by the AP-1 complex Nucl Acids Res 21: 1873–1879

13 Chada K, Magram J, Raphael K, Radice G, Lacy E, Constantini F (1985) Specific expression of foreign beta globin gene in erythroid cells of transgenic animals. Nature 314: 377–380

14 deChiara TM, Efstratiadis A, Robertson EJ (1991) Parental imprinting of the mouse insulin-like growth factor gene. Cell 64: 849–859

15 deChiara TM, Robertson EJ, Efstratiadis A (1990) A growth deficiency phenotype in heterozygous mice carrying an insulin-like growth factor II gene disrupted by targetting. Nature 345: 78–80

16 Doi T, Striker LJ, Gibson CC, Agodoa LY, Brinster RL, Striker GE (1990) Glomerular lesions in mice transgenic for growth hormone and insulin like factor-I. Am J Path 137: 541–552

17 Dressler GR, Deutsch U, Chowdbury K, Nornes HO Gruss P (1990) Pax-2, a new murine paired box containing gene and its expression in the developing secretory system. Development 109: 787–795

18 Dressler GK, Douglas EC (1992) Pax-2 is a ANA-binding protein expressed in embryonic kidney and Wilms tumour. Proc Natl Acad Sci USA 89: 1179–1183

19 Dressler GR, Wilkinsson JE, Rothenpieler UW, Patterson LT, Williams L, Westphal H (1993) Deregulation of pax-2 expression in transgenic mice generates severe kidney abnormalities. Nature 362: 65–67

20 Edwards RH, Rutter WJ, Hanahan D (1989) Directed expression of NGF to pancreatic beta cells in transgenic mice leads to selective hyperinnervation of the islets. Cell 58: 161–170

21 Ellis C (1990) Studies of the roles of IGF-II during mouse development. DPhil Thesis Oxford University

22 Engström W, Lindham S, Schofield PN, Wiedemann Beckwith Syndrome. Eur J Pediatr 147: 450–457

23 Feinberg AP (1993) Genomic imprinting and gwnw activation in cancer. Nature Genet 4: 110–113

24 Ferguson-Smith AC, Sasaki H, Cattanach BM, Surani MA (1993) Parental-origin-specific epigenetic modification of the mouse H19 gene. Nature 362: 751–755

25 Fitson A, Louvi A, Efstradiatis A, Robertson EJ (1993) Rescue of the T-associated maternal effect in mice carrying null mutations in IGF-2 and IGF-2-r, two reciprocally imprinted genes. Development 118: 731–736

26 Fromont-Racine M, Bucchini D, Madsen O, Desbois D, Linde S, Nielsen J, Saulnir C, Ripoche M-A, Jami J, Picet R (1990) Effect of 5'-flanking sequence deletions on expression of the human insulin gene in transgenic mice. Mol Endocrinol 4: 669–677

27 Fukamizu A, Seo MS, Hatae T, Yokohama M, Nomura T, Katsuki K and Murakami K (1990) Tissue specific expression of the human renin gene in transgenic mice. Biochem Biophys Comm 165: 826–832

28 Gardner RL (1968) Mouse chimaeras obtained by the injection of cells into the blastocyst. Nature 220: 596–597

29 Giannourakis N, Deal C, Paquette J, Goodyear CG, Polychronakas C (1993). Parental genomic. imprinting of the human IGF2 gene. Nature Genet 4: 98–101

30 Gordon JW, Scangos GA, Plotkin DJ, Barbariosa JA, Ruddle FH (1980) Genetic transformation of mouse embryos by microinjection of foreign DNA. Proc Natl Acad Sci USA 77: 7380–7384

31 Gordon JW, Ruddle FH (1981) Intergration and stable germ line transmission of genes injected into mouse pronuclei. Science 214: 1244–1246

32 Gordon JW (1989) Transgenic Animals. Int Rev Cytol 115: 171–229

33 Haig D, Graham C (1991) Genomic imprinting and the strange case of the insulin-like growth factor II receptor. Cell 64: 1045–1046

34 Haig D, Westoby M (1989) Parent-specific gene expression and the triploid endosperm. Am. Nat 134: 147–155

35 Harris S, McClenaghan JP, Simons JP, Ali S, Clark AJ (1990) Gene expression in the mammary gland. J Reprod Fert 88: 707–715

36 Henry B-PC, Chenbensse V, Beldjord C, Schwartz C, Utermann G, Junien C (1991) Uniparental patenal disomy in a genetic cancer disposing syndrome. Nature 351: 665–667

37 Hilberg F, Wagner EF (1992) Embryonic stem cells lacking functional c-jun. Consequences for growth and differentiation, AP-1 activity and tumorigenicity. Oncogene 7: 2371–2380

38 Hiberg F, Aguzzi A, Howells N, Wagner EF (1993) C-jun is essential for normal mouse development and hepatogenesis. Nature 365: 179–181

39 Hogan B, Constantini F, Lacy E (1986) Manipulating the mose embryo a laboratory manual. Cold Spring Harbor Press, Cold Spring Harbor, New York

40 Hooper ML (1992) Embryonal stem cells: introducing planned chances into the animal germ line. In "Modern Genetics" Harwood Academic Publishers, Switzerland

41 Jaenisch R, Mintz B (1974) Simian virus 40 DNA sequences in DNA of healthy adult mice derived from preimplantation blastocysts injected with viral DNA. Proc Natl Acad Sci USA 71: 1250–1255

42 Jaenisch R (1976) Germ line integration and mendelian transmission of the exogenous Moloney leukemia virus. Proc Natl Acad Sci USA 73: 1260–1264

43 Joyner AL (1993) Gene targeting: a practical approach. Oxford University Press, Oxford

44 Junien C (1992) Beckwith-Wiedemann syndrome, tumourigenesis and imprinting. Curr Opin Gen Dev 2: 431–438

45 Kajimoto Y, Kawamori R, Umayahara Y, Iwama N, Imano E, Morishima T, Yamasaki Y, Kamada T (1993) An AP-1 enhancer mediates TPA-induced transcriptional activation of the chicken insulin-like growth factor I gene. Biochem Biophys Res Comm 190: 767–773

46 Koopman P, Gubbay J, Collignon J, Lovell-Badge R (1989) Zfy gene expression patterns are not compatible with a primary role in mouse sex determination. Nature 342: 940–942

47 Koopman P, Gubbay J, Vivian N, Goodfellow P, Lovell-Badge R (1991) Male development of chromosomally female mice transgenic for Sry. Nature 351: 117–121

48 Lang RA, Metcalf D, Cuthbertson RA, Lyons I, Stanley E, Kelso A, Kannourakis G, Williamsson DJ, Klintworth GK, Gonda TJ, Dunn A (1987) Transgenic animals expressing a hematopoetic growth factor gene develop accumulation of macrophages, blindness and a fatal syndrome of tissue damage. Cell 51: 675–686

49 Lee JE, Tantravahi U, Boyle AL, Efstratiadis A (1993) Parental impriting of an IGF-2 transgene. Mol Rep Dev 35: 382–390

50 Li JE, Beard C, Jaenish R (1993) Role for DNA methylation in genomic impriting. Nature 366: 362–365

51 Little M, van Heyningen, Hastie N (1991) Dads and disomy and disease. Nature 351: 609–610

52 Liu J, Baker J, Perkins AS, Robertson LJ, Efstratiadis A (1993) Mice carrying null mutations of the genes encoding insulin like growth factor 1 and type 1 IGF receptor. Cell 75: 59–72

53 Mansour SL, Thomas KR, Capecchi M (1988) Disruption of the proto-oncogene int-2 in mouse embryo-derived stem cells: a general strategy for targeting mutations to non-selectable genes. Nature 336: 348–353

54 Marban SL, DeLoia JA, Gearhart JD (1988) Hyperinsulinemia in transgenic mice carrying multiple copies of the human insulin gene Dev Gene 10: 356–364

55 Mason AJ, Pitts SL, Nikolics K, Szonyi E, Wilcox JN, Seeberg PH, Stewart TA (1986) The hypogonadal mouse. Reproductive functions restored by gene therapy. Science 234: 1372–1378

56 Mathews LS, Hammer RE, Behringer R, d'Ercole AJ, Bell CI, Brinstcr R, Palmiter RD (1988) Growth enhancement of transgenic mice expressing human insulin-like growth factor 1. Endocrinology 123: 2827–2833

57 Mathews LS, Hammer RE, Brinster RL Palmiter RD (1988) Expression of insulin-like growth factor 1 in transgenic mice with elevated levels of growth hormone is correlated with growth. Endocrinology 123: 433–437

58 Meisler M (1992) Insertional mutations of classical and novel genes in transgenic mice. Trends Genet 8: 341–344

59 Mintz B (1962) Formation of genotypically mosaic mouse embryos. Am Zool 2: 432 (Abstract)

60 Ogawa O, Eccles J, Szeto J, McNoe LA, Dun K, Maw MA, Smith PJ, Reeve AE (1993) Relaxation of insulin like growth factor II gene imprinting implicated in Wilms' tumour. Nature 366: 749–751

61 Ohlsson R, Nyström A, Pfeifer-Ohlsson S, Tohonen V, Hedberg F, Schofield PN, Flarn F, Ekström T (1993) *IGF2* is parentally imprinted during human embryogenesis and in the Beckwith-Wiedemann syndrome. Nature Genet 4: 95–97

62 Palmiter RD, Brinster RL (1985) Transgenic mice. Cell 41: 343–345

63 Palmiter RD, Brinster R (1986) Germ line transformation of mice. Ann Rev Genet 29: 233–236

64 Palmiter RD, Brinster RL, Hammer RE, Trumbauer MG,

Rosenfeld MG, Birnberg NC, Evans RM (1982) Dramatic growth of mice that develop from eggs microinjected with metallothionein-growth hormone fusion genes. Nature 300: 611–615

65 Palmiter RD, Norstedt G, Gelinas RE, Hammer RE, Brinster RL (1983) Meallothionein-human GH fusion genes stimulate growth of mice. Science 222: 809–814

66 Quaife CJ, Mathwes LS, Pinkel C, Hammer RE, Brinster RL, Palmiter RD (1989) Histopathology associated with elevated levels of growth hormone and insulin like growth factor I in transgenic mice. Endocrinology 124: 40–48

67 Rainier S, Johnson LA, Dobry CJ, Ping AJ, Grundy PE, and Feinberg AP (1993) Relaxation of imprinted genes in human cancer. Nature 362: 747–749

68 Robertson EJ (1991) Using embryonic stem cells to introduce mutations into the mouse germ line. Biol Reprod 44: 238–245

69 Sasaki H, Jones PA, Chaillet JR, Ferguson-Smith AC, Barton SC, Reik W, Surani MA (1992) Parental imprinting; potentially active chromatin of the repressed maternal allele of the mouse insulin-like growth factor II gene. Genes Dev 6: 1843–1856

70 Schoenle E, Zapf J, Humbel R, Froesch E (1982) IGF-I stimulates growth in hypophysectomized rats. Nature 296: 252–253

71 Schofield PN, Lindham S, Engström W (1989) Analysis of gene dosage on chromosome 11 in children suffering from Beckwith Wiedemann Syndrome. Eur J Pediatr 148: 320–324

72 Schofield PN (1992) The insulin-like growth factors structure and biological functions. Oxford University Press, Oxford

73 Schofield PN, Engström W (1992) Insulin like growth factors in human cancer. In "The insulin-like growth factors structure and biological functions" Oxford University Press, Oxford pp. 240–257

74 Selden RF, Skoskicwicz MZ, Burke Howie K, Russell PS, Goodman HM (1986) Regulation of human insulin gene expression in transgenic mice. Nature 321: 525–528

75 Sigmund CD, Jones CA, Mullins JJ, Kim U, Gross K (1990) Expression of murine cutaneous renin genes in subcutaneous connective tissue. Proc Natl Acad Sci USA 87: 7993–7997

76 Sigmund CD, Jones CA, Fabian JR, Mullins JJ, Gross KW (1990) Tissue and cell specific expression of a renin promoter reporter gene construct in transgenic mice. Biochem Biophys Comm 170: 344–350

77 Smeyne RJ, Vendrell M, Hayward M, Baker SJ, Miao GG, Schilling K, Robertson LM, Curran T, Morgan JI (1993) Continuous c-fos expression precedes programmed cell death in vivo. Nature 363: 166–169

78 Sola C, Tronik D, Dreyfus M, Babinet C, Rougeon F (1989) Renin promoter SV40 Large T antigen transgenes induce tumors irrespective of normal cellular expression of renin genes. Oncogene Res 5: 149–153

79 Spaventi R, Antica M, Pavclic K (1990) Insulin and insulin-like growth factor I (IGF-I) in early mouse embryogenesis. Development 108: 491–495

80 Stewart TA, Clift S, Pitts-Meek S, Martin L, Terrell G, Liggitt D, Oakley H (1992) An evaluation of the functions of the 22-kilodalton (kDa) the 20-kDa, and the N-terminal polypeptide forms of human growth hormone using transgenic mice. Endocrinology 130: 405–414

81 Stoger R, Kubicka P, Liu C-G, Kafri T, Razin A, Cedar H, Barlow DP (1993) Maternal-specific methylation of the imprinted mouse locus identifies the expressed locus as carrying the imprinted signal. Cell 73: 61–71

82 Suematsu S, Matsuda T, Aozasa K, Akira S, Nakano N, Ohno S, Yamamura J, Hirnao T, Kishimoto T (1989) IgG plasmocytosis in IL-6 transgenic mice. Proc Natl Acad Sci USA 86: 7547–7551

83 Surani MA (1993) Silence of the genes. Nature 366: 302–303

84 Surani MA, Kothary R, Allen ND, Singh P, Fundele R, Ferguson-Smith AC, Barton SC (1990) Genomic imprinting and development in the mouse. Development (suppl) 89–98

85 Surani MA, Reik W, Norris MI, Barton SC (1986) Influence of germline modifications of homologous chromosomes on mouse development. J Embryol Exp Morphol 97 (suppl) 123–126

86 Tarkowski K (1961) Mosue chimaeras developed from fused eggs. Nature 190: 857–860

87 te Riele H, Maandag ER, Berns M (1992) Highly efficient gene targeting in embryonic stem cells through homologous recombination. Proc Natl Acad Sci USA 89: 5128–5132

88 Van Deursen J, Wieringa B (1992) Targeting of creatin kinase M gene in embryonic stem cells uisng isogenic and nonisogenic vectors. Nucleic Acids Res 20: 3815–3820

89 Van Heyningen V, Hastie ND (1992) Wilms' tumour: reconciling genetics and biology. Trends Genet 8: 16–21

90 Ward A, Ellis CJ (1992) The insulin like growth factor genes. In "Insulin like growth factors - Structure and function" Oxford University Prees

91 Wilde CJ, Clark AJ, Kerr MA, Knight CH, McCleanaghan M, Simons P (1992) Mammary development and milk secretion in transgenic mice expressing the sheep beta lactoglobulin gene. Biochem J 284: 717–720

92 Zemel S, Bartolomei MS, Tilghman SM (1992) Physical linkage of two mammallan imprinted genes, H19 and insulin-like growth factor 2. Nature Genet 2: 61–65

93 Zhang Y, Tycko B (1992) Monoallelic expression of the human H19 gene. Nature Genet 1: 40–44